STANDARD OPERATING PROCEDURE FOR: PFAS in 6-dpf Zebrafish Larvae by UPLC-MS/MS

SOP No. E-ADH-RCU-SOP-3364-1; Effective date: 9/06/2018

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1. Scope of Application

This standard operating procedure (SOP) shall provide procedures for the quantitative analysis of 8 per and poly fluorinated alkyl substances (PFAS) in 6-day postfertalization (dpf) zebrafish larvae (ZFL) prepared by protein precipitation and analyzed by Thermo Vanquish ultra-performance liquid chromatography (UPLC) and Orbitrap Fusion mass spectrometer (MS/MS). Refer to Table 1 for the 8 PFAS included in the SOP. This SOP applies only to a Thermo Vanquish UPLC Orbitrap Fusion MS/MS having a mass axis calibrated per the manufacturer's specifications and operating with the optimized parameters listed in Section 6.1.3, UPLC-MS/MS Method.

Chemical name	CAS#	MW	Highest Dose that will be used for Testing
GenX (free acid; Undecafluoro-2-methyl-3-oxahexanoic acid)	13252-13-6	330.053	80[HYPERLINK "http://www.sigmaaldrich.com/catalog/Lookup.do?N5=CAS+No.&N3=mode+matchpartialmax&N4=2043-57-4&D7=0&D10=&N25=0&N1=S_ID&ST=RS&F=PR"]
Gen X (ammonium salt; Ammonium perfluoro(2-methyl-3-oxahexanoate))	62037-80-3	347.084	80[HYPERLINK "http://www.sigmaaldrich.com/catalog/Lookup.do?N5=CAS+No.&N3=mode+matchpartialmax&N4=2043-57-4&D7=0&D10=&N25=0&N1=S_ID&ST=RS&F=PR"]
Perfluorooctanesulphonic acid (PFOS)	1763-23-1	500.126	80[HYPERLINK "http://www.sigmaaldrich.com/catalog/Lookup.do?N5=CAS+No.&N3=mode+matchpartialmax&N4=2043-57-4&D7=0&D10=&N25=0&N1=S_ID&ST=RS&F=PR"]
Perfluorooctanoic acid(PFOA)	335-67-1	414.07	80[HYPERLINK "http://www.sigmaaldrich.com/catalog/Lookup.do?N5=CAS+No.&N3=mode+matchpartialmax&N4=2043-57-4&D7=0&D10=&N25=0&N1=S_ID&ST=RS&F=PR"]
Perfluorohexanoic Acid (PFHxA)	307-24-4	314.054	80[HYPERLINK "http://www.sigmaaldrich.com/catalog/Lookup.do?N5=CAS+No.&N3=mode+matchpartialmax&N4=2043-57-4&D7=0&D10=&N25=0&N1=S_ID&ST=RS&F=PR"]
Perfluorohexane sulfonic	3871-	438.	80[HYPERLINK "http://www.sigmaaldrich.com/catalog/Lookup.do?N5=CAS+No.&N3=mode+matchpartialmax&N4=2043-57-

acid (PFHxS)	99-6	201	4&D7=0&D10=&N25=0&N1=S_ID&ST=RS&F=PR"]
4,8-dioxa-3H-perfluorononanoate (ADONA)	958445-44-8	400.05	80 µM
Nafion, Compound 1	31175-20-9	544.135	80 µM

2. Method Summary

10, 6-dpf ZFL are homogenized using zirconia/silica beads and a FastPrep homogenizer in 0.1 M formic acid fortified with a surrogate. Protein is precipitated from the homogenate with acetonitrile containing internal standards and clarified with a centrifuge. The extract is diluted with aqueous 0.4 mM ammonium formate for an LC extract. The LC extract is analyzed with UPLC- Orbitrap MS with a C18 column. Concentration for each PFAS analyte is determined by internal standard technique using isotopically labeled internal standards and matrix-matched calibration standards. Identification for each target analyte in full scan MS1 is based on accurate mass (m/z with 10 ppm window) of the $[M-H]^-$, relative retention time, and MS1 peak abundance ratio of $[M-H]^-$ to a source decomposition product.

The performance characteristics of the analysis method for the 8 analytes in 6-dpf ZFL was demonstrated and documented to be suitable and reliable for the intended application by the analysis of a method validation set. Refer to Appendix 9.1, Method Validation Results Report for Method Detection Limits (MDL), calibration range, and performance data.

3. Definitions and Acronyms

3.1. ACN – Acetonitrile.

3.2. ACS – American Chemical Society.

3.3. Calibration Standard (CS) - A solution of target analytes, internal standards, and the surrogate analyte in solvent used to demonstrate that the relationship between instrument response and concentration is continuous and reproducible for each analyte of interest.

3.4. Chain of Custody (COC) – A Document used to record the transfer of samples between parties.

3.5. FA – Formic Acid.

3.6. HPLC - high performance liquid chromatography system.

3.7. Internal Standard (IS) Response - Peak area for each internal standard used to monitor for gross failures during sample preparation and instrument performance.

3.8. ISHEM - Initial Safety Health and Environmental Management.

3.9. kV – Killivolts.

3.10. L – Liter.

3.11. Laboratory Reagent Blanks (LRB) – Reagent water processed as a sample used to demonstrate that reagents and analysis system are free from contamination.

3.12. LC/MS – Liquid chromatography mass spectrometry.

3.13. LC/MSMS – Liquid chromatography tandem mass spectrometry.

3.14. Lower Limit of Quantification (LLOQ) – The concentration of the lowest CS used in the analysis of a batch reported in dilution corrected units.

3.15. mL – Milliliter.

3.16. mM – Millimolar.

3.17. mm – Millimeter.

3.18. M/N – Manufacturer number.

3.19. m/z – Mass to charge ratio.

3.20. MeOH – Methanol.

3.21. Method Blanks (MB) - 10, 6-dpf ZFL prepared and analyzed with internal standards as a sample. MB control samples are used to demonstrate that the entire preparation and analysis system is free from contamination.

3.22. Method Detection Limit (MDL) - The minimum concentration of a substance that can be measured and reported with 99% confidence that the analyte is greater than zero for an analyte in a given matrix.

3.23. MRM – Multiple reaction monitoring.

3.24. NA – Not Applicable.

3.25. ng/mL – nanogram per milliliter.

3.26. NH_4 Formate – Ammonium Formate.

3.27. NERL – National Exposure Research Laboratory.

3.28. NHEERL - National Health and Environmental Effects Research Laboratory.

3.29. ORD – Office of Research and Development.

3.30. pg/mL – Picogram per milliliter.

3.31. P/N – Part number.

3.32. PFAS - Per and poly fluorinated alkyl substances.

3.33. QA – Quality assurance.

3.34. QC – Quality Control.

3.35. Quality Control (QC) Standard – 10, 6-dpf ZFL spiked with target analytes. QC standards are used to demonstrate the continued acceptance of the calibration and assess the method's accuracy and precision.

3.36. Qualifier/Quantifier (Qual/Quan) Ion Ratio – The peak area of the qualifier ion divided by the peak area of the quantifier ion. The Qual/Quan ion ratio is used for qualitative identification of the target analyte.

3.37. Relative Retention Time (RRT) Ratio –Ratio of the retention time of the internal standard quantifier ion divided by the retention time of the target analyte quantifier ion. RRT is used to assign a qualitative identification.

3.38. Retention Time (RT) - time taken for compound to pass through a chromatography column.

3.39. S/N – Serial number.

3.40. Solvent Blanks (SB) – An aliquot containing MeOH, ACN, formic acid in 0.4 mM aqueous NH_4 Formate an LC vial used equilibrate the UPLC- Orbitrap.

3.41. SHEM - Safety Health and Environmental Management.

3.42. SOP - Standard Operating Procedure.

3.43. Surrogate Analyte - Perfluorononanoic Acid (PFNA) is included in preparation of the LRB, MB, and all samples. The surrogate accuracy is used to demonstrate acceptable sample preparation and analysis performance.

3.44. UPLC - Ultra-high performance liquid chromatography system.

3.45. Upper Limit of Quantification (ULOQ) – The concentration of the highest CS used in the analysis of a batch reported in dilution corrected units.

3.46. UV – ultraviolet.

3.47. V – Volts.

3.48. μL – Microliter.

3.49. μM – Micromolar

4. Prerequisites

4.1. Equipment and Supplies

4.1.1. Equipment/Instruments

4.1.1.1. Balance, AE100 Analytical Balance (Mettler Toledo) S/N P00633 located in D377-A or equivalent.

4.1.1.2. Vortex Genie (Scientific Industries, Bohemia, NY) M/N 6560 (S/N 2-415260) or equivalent.

4.1.1.3. FastPrep 24 Homogenizer, (MP Biomedicals, Santa Ana, CA) M/N FastPrep 24 (S/N 6110188).

4.1.1.4. Refrigerated Centrifuge, Jouan, M/N BR4 (S/N 403110090).

4.1.1.5. Pipettes, Rainin Pipette-Lite XLS+ L10 (S/N B619470071), L20 (S/N G1482996T), L200 (S/N G1482996T), L1000 (S/N C15695857) or equivalents.

4.1.1.6. Vanquish (UPLC) ultra-high performance liquid chromatography system (Thermo Electron, Waltham, MA) with Vanquish UPLC Pump (S/N 8304440), Vanquish UPLC Autosampler (S/N 8304448) and Vanquish UPLC Column Compartment (S/N 65003100).

4.1.1.7. Orbitrap Fusion mass spectrometer (Thermo Electron Corporation, Waltham, MA; S/N EXRSN10137).

4.1.1.8. Xcalibur 4.131.9 software (Thermo Electron Corporation, Waltham, MA).

4.1.2. Chemical Reagents

4.1.2.1. Water, HPLC Grade or better, Fisher Scientific P/N W6-4 or equivalent.

4.1.2.2. Acetonitrile UV, High Purity Solvent, Burdick & Jackson P/N 015-4 or equivalent.

4.1.2.3. Methanol, Certified ACS or better, Fisher Scientific P/N A412-4 or equivalent.

4.1.2.4. Ammonium Formate, >99%, Sigma P/N 70221 or equivalent.

4.1.2.5. Perfluoro-n-hexanoic acid (PFHxA), 97%, neat, SynQuest P/N 2121-3-39 or equivalent.

4.1.2.6. Perfluoro-n-[1,2-$^{13}C_2$]hexanoic acid, 1.2 mL of 50 µg/mL PFHxA-$^{13}C_2$, 98% purity in methanol MeOH / Water (< 1%), Wellington Laboratories P/N MPFHxA or equivalent.

4.1.2.7. Perfluorohexanesulfonic acid potassium salt (PFHxS Potassium Salt), >98%, neat, SynQuest P/N 6164-3-X4 or equivalent.

4.1.2.8. Sodium perfluoro-1-[1,2,3-$^{13}C_3$]-hexanesulfonate, 1.2 mL of 50 µg/mL PFHxS-$^{13}C_3$, 98% purity in methanol MeOH / Water (< 1%), Wellington Laboratories P/N MPFHxS or equivalent.

4.1.2.9. Perfluoro-n-octanoic acid (PFOA), 96%, neat, SynQuest P/N 2121-3-18 or equivalent.

4.1.2.10. Perfluoro-n-[1,2,3,4-$^{13}C_4$]octanoic acid, 1.2 mL of 50 µg/mL PFOA-$^{13}C_4$, 98% purity in methanol MeOH / Water (< 1%), Wellington Laboratories P/N MPFOA or equivalent.

4.1.2.11. Perfluoro-1-octanesulfonic acid (PFOS), >98%, neat, SynQuest P/N 6164-3-08 or equivalent.

4.1.2.12. Sodium perfluoro-1-[1,2,3,4-$^{13}C_4$]-octanesulfonate, 1.2 mL of 50 µg/mL PFOS-$^{13}C_4$, 98% purity in methanol MeOH / Water (< 1%), Wellington Laboratories P/N MPFOS or equivalent.

4.1.2.13. 2,3,3,3-Tetrafluoro-2-(1,1,2,2,3,3,3-heptafluoropropoxy)propanoic acid (GenX), 1.2 mL of 50 µg/mL GenX, 98% purity in methanol MeOH / Water (< 1%), Wellington Laboratories P/N HFPO-DA or equivalent.

4.1.2.14. 2,3,3,3- Tetrafluoro-2-(1,1,2,2,3,3,3-heptafluoropropoxy)-$^{13}C_3$-propanoic acid, 1.2 mL of 50 µg/mL $^{13}C_3$-GenX, 98% purity in methanol MeOH / Water (< 1%), Wellington Laboratories P/N M3HFPO-DA or equivalent.

4.1.2.15. NaDONA (ADONA), 1.2 mL of 50 µg/mL ADONA 98% purity in methanol MeOH / Water (< 1%), Wellington Laboratories P/N NaDONA or equivalent.

4.1.2.16. Nafion, Compound 1, The Chemours Company, 98%, P/N NA.

4.1.2.17. Perfluorononanoic Acid (PFNA) (surrogate analyte), 97% neat solid, Aldrich P/N 394459-5G or equivalent.

4.1.2.18. Perfluoro-n-[1,2,3,4,5-$^{13}C_5$]nonanoic acid, 1.2 mL of 50 µg/mL PFNA-$^{13}C_5$, 98% purity in methanol MeOH / Water (< 1%), Wellington Laboratories P/N MPFNA or equivalent.

4.1.2.19. Formic Acid (FA), >99%, Thermo Scientific Pierce P/N 28905 or equivalent.

4.1.3. Supplies

4.1.3.1. 2 mL Microcentrifuge Tubes, Fisherbrand™ Nonsterile Threaded End Microcentrifuge Tubes without Caps, Fisher P/N 02-681-344.

4.1.3.2. 2 mL Microcentrifuge Tube Screw Caps, Fisherbrand™ Colored Screw Caps, Fisher P/N 02-681-368.

4.1.3.3. 1.0 mm diameter (Dia) Zirconian/Silica Beads, BioSpec Products P/N 11079110Z.

4.1.3.4. Falcon™ 15mL Conical Centrifuge Tubes (polypropylene), Corning, Inc. P/N 352196, 15 mL conical centrifuge tube, polypropylene, Fisher P/N 14-959-53A.

4.1.3.5. LC vials, 500 µL Polypropylene LVV 9 mm THD, Laboratory Supply Distributor P/N 30509P-1232.

4.1.3.6. LC vial Caps, 9 mm PTFE/Sil, Laboratory Supply Distributor P/N 2150BL-10.

4.1.3.7. Disposable polypropylene pipette tip (1-10 µL), 20µl Olympus Ergo X Pipet Tip for Rainin LTS, Genesee Scientific P/N 24-721R.

4.1.3.8. Disposable polypropylene pipette tip (20 - 200 µL), 250µl Olympus Ergo

X Pipet Tip for Rainin™ LTS, Genesee Scientific P/N 24-750R.

4.1.3.9. Disposable polypropylene pipette tip (100 -1000 µL), Tips LTS 1 mL 768/8 RT-L1000, Genesee Scientific P/N 24-760R.

4.1.3.10. UPLC Column, ACQUITY UPLC BEH C18 Column, 130Å, 1.7 µm, 2.1 mm X 50 mm, Waters Corp P/N 186002350.

4.1.3.11. Holdup Column, XBridge BEH C18 Column, 130Å, 3.5 µm, 2.1 mm X 50 mm, 1/pkg, Waters Corp P/N 186003021.

4.2. Training Requirements

4.2.1. The individual performing this protocol must have completed the ORD Safety Health and Environmental Management (SHEM) Initial Safety Health and Environmental Management (ISHEM) course and be in compliance with SHEM annual safety training requirements.

4.2.2. The individual performing this protocol must also have completed the mandatory ORD QA training, and be in compliance with the once every three years thereafter QA Refresher Course requirement.

4.2.3. The individual performing this protocol should have a background in science, general laboratory experience, and routine experience operating LC/MSMS instrumentation.

4.2.4. The individual performing this protocol must be trained on the operation and routine maintenance of the Thermo Vanquish (UPLC) and Orbitrap Fusion mass spectrometer as certified by ORD Certification Statement for Demonstration of Capability.

5. Cautionary Notes or Special Considerations

5.1. Laboratory containers must be polypropylene to avoid sorption and loss of perfluorinated chemicals.

5.2. Low binding pipette tips and tubes must be avoided as their coatings are often a source of perfluorinated compounds that can add significant contamination.

5.3. Supplies and reagents must be verified to be free of the 8 target per and poly fluorinated chemicals listed in Section 1 of this SOP by the analysis of solvent blanks and laboratory reagent blanks.

6. Procedure/Method

6.1. Steps to be Taken

6.1.1. Verification of Pipettes

6.1.1.1. Fill a container with de-ionized water and allow it to equilibrate to room temperature for a minimum of 1 hour in the same laboratory with the analytical balance.

6.1.1.2. Place a small container on the analytical balance and tare.

6.1.1.3. With the pipette set to its mid-range volume (pipette delivering volumes > 20 µL) or max volume (pipettes delivering volumes < 20 µL), aspirate an aliquot of DI water from the sample aliquot container and dispense into the container on the balance.

6.1.1.4. Record the mass and tare the balance.

6.1.1.5. Repeat steps 6.1.1.3 and 6.1.1.4 for 5 total replicates.

6.1.1.6. Calculate the volume delivered from weights using the density of water, the average and % RSD for the calculated volumes delivered, and the % Differences for the average volume delivered.

6.1.1.7. Compare the calculated results to QC metrics listed in section 6.3.1 and take any corrective actions listed in section 6.4.1 as appropriate.

6.1.2. Preparation of Solutions, Standards, and Controls

6.1.2.1. Prepare Solutions

6.1.2.1.1. 0.4 mM NH_4-Formate in Reagent Water with 5% Acetonitrile (UPLC Mobile Phase A)

Weigh 25.2 mg (+/-5 mg) ammonium formate to weigh paper. Transfer to 1 liter (L) reservoir dissolve in water. Add 50 mL ACN. Dilute to 1 L with reagent water. Cap and mix by inversion. Sonicate for 15 minutes to degas.

6.1.2.1.2. 0.4 mM NH_4-Formate in Acetonitrile with 5% Reagent Water (UPLC Mobile Phase B)

Weigh 25.2 mg (+/-5 mg) ammonium formate to weigh paper. Transfer to 1-liter (L) reservoir and dissolve in 50 mL of reagent water. Dilute to 1 L with ANC. Cap and mix by inversion. Sonicate for 15 minutes to degas.

6.1.2.1.3. 0.1 M Formic Acid in Reagent Water

Add 943 µL formic acid to reagent water in 250 mL volumetric flask and dilute to volume with reagent water. Invert to mix. Transfer to 250 mL bottle.

6.1.2.1.4. 0.4 mM NH_4-Formate in Reagent Water

Weigh 25.2 mg (+/-5 mg) ammonium formate to weigh paper. Transfer to 1-liter (L) reservoir dissolve in water. Dilute to 1 L with reagent water. Cap and mix by inversion.

6.1.2.1.5. Prepare 2.5 M NaOH in reagent water.

WARNING

Concentrated NaOH slowly dissolves glass and gets contaminated with silica salts. Prepare 2.5 M NaOH solutions no more than 3 days in advance of its use for stock preparation.

Weigh ~50 g of NaOH to 500 mL bottle. Add 200 mL of water and stir to dissolve. Dilute to 500 mL with water, cap and mix.

6.1.2.2. Prepare Stocks from Neat Materials in MeOH with 5% aqueous 2.5 M NaOH. The addition of NaOH prevents the formation of methyl esters of PFAS carboxylic acids and thus loss of analyte from stock Methanolic solutions.

Weigh ~5 - 10 mg of PFHxA, PFHxS, PFOA, PFOS, PFNA, GenX, and Nafion Compound 1 to individual 5 mL volumetric flasks (VF). Dissolve in ~1 - 2 mL of MeOH. Add 250 µL of aqueous 2.5 M NaOH. Mix and dilute to volume with MeOH. Transfer to labelled plastic tube and store at room temperature away from light. The stocks are stable for a minimum of 3 months.

6.1.2.3. Prepare Mixed Intermediate Standards and Surrogate Spiking Solution.

6.1.2.3.1. Prepare Internal Standard Mixed Intermediate Standard - 1 (ISMIS-1) at 62.5 ng/mL from stocks of $^{13}C_2$-PFHxA, $^{13}C_3$-PFHxS, $^{13}C_4$-PFOA, $^{13}C_4$-PFOS, and $^{13}C_3$-GenX by dilution with MeOH. Prepare a volume of ISMIS-1 > 500 µL + 400 µL * the total number of blanks, standards, controls, and samples.

6.1.2.3.2. Prepare Native Mixed Intermediate Standard - 1 (NMIS-1) at ~ 50 µg/mL from ~1 mg/mL stocks of PFHxA, PFHxS, PFOA, PFOS, GenX, PFESA1, and PFNA in MeOH (refer to 6.1.2.2 for stock solution preparation).

6.1.2.3.3. Prepare Native Mixed Intermediate Standard - 2 (NMIS-2) at ~ 5 μg/mL PFHxA, PFHxS, PFOA, PFOS, GenX, PFESA1, PFNA, and ADONA by dilution of 100 μL of NMIS-1 and 100 μL of the 50 μg/mL ADONA stock with 800 μL of MeOH.

6.1.2.3.4. Prepare Surrogate Spiking Solution (PFNA-1) at ~ 250 ng/mL from the ~1 mg/mL stock by dilution with 0.1 M aqueous formic acid.

6.1.2.4. Preparation of Blanks

6.1.2.4.1. Solvent Blanks (SB) - Combine 25 μL of MeOH, 75 μL of 0.1 M FA in reagent water, and 400 μL of ACN. Dilute 50 μL with 200 μL of 0.4 mM NH4 Formate in LC vial. Prepare enough SB to be run at the beginning and end of each batch and after every QC.

6.1.2.4.2. Laboratory Reagent Blanks (LRB) - Add 100 μL of surrogate spiking solution PFNA-1 to 400 μL of ISMIS-1 in 1.5 mL tube and vortex to mix. Dilute 50 μL with 200 μL of 0.4 mM NH_4 Formate in LC vial.

Note – Prepare sufficient quantity to generate the number of LRB-LC Extracts equal to 10% of the total number of samples to be run.

6.1.2.4.3. Blank Matrix (BM) - Remove as much water as possible from 2 mL tube containing 10, 6-dpf ZFL. Add ~ 100 mg (10 - 15 beads) 1.0 mm diameter (DIA) zirconia/silica beads to the 2 mL tube. Add 100 μL PFNA-1. Homogenize by 2, 20 second bursts at 6 m/s using FastPrep homogenizer. Dilute with 400 μL of ACN and vortex (protein precipitation). Centrifuge at 4 °C and 14,000 rpm for 15 minutes. Dilute 50 μL of the protein precipitation supernatant with 200 μL of aqueous 0.4 mM NH_4-Formate in LC vial.

6.1.2.4.4. Method Blank (MB) - Remove as much water as possible from 2 mL tube containing 10, 6-dpf ZFL. Add ~ 100 mg (10 - 15 beads) 1.0 mm diameter (DIA) zirconia/silica beads to 2 mL tube. Add 100 μL 0.1 M aqueous formic acid. Homogenize by 2, 20 second bursts at 6 m/s using FastPrep homogenizer. Dilute with 400 μL of ISMIS-1 and vortex (protein precipitation). Centrifuge at 4 °C and 14,000 rpm for 15 minutes. Dilute 50 μL of the protein precipitation supernatant with 200 μL of aqueous 0.4 mM NH_4-Formate in LC vial.

Note – Prepare sufficient quantity to generate the number of MBs equal to 10% of the total number of samples to be run.

6.1.2.5. Preparation of Matrix-matched Calibration and Quality Control Standards

6.1.2.5.1. Prepare 6-dpf ZFL Homogenate – Prepare sufficient volume of 6-dpf ZFL homogenate in 0.1 M aqueous formic acid at approximately 10 ZFL per 75 µL 0.1 M aqueous formic acid to allow preparation of matrix-matched calibration and quality control standards.

6.1.2.5.2. Matrix-matched Calibration Standards (CS) – Prepare a minimum of 7, calibration standards covering the concentration range of 10 ng/mL – 1.25 µg/mL (20 ng/mL – 2.5 µg/mL GenX) in 100 µL homogenate. Prepare 7 serial dilutions (SD 1 - 7) of NMIS-2 by diluting 500 µL to 1000 µL with MeOH in 1.5 mL tube. Add 25 µL of each SD and NMIS-2 to 75 µL of the 6-dpf ZFL homogenate and vortex to mix. Dilute with 400 µL of ISMIS-1 and vortex (protein precipitation). Centrifuge at 4 °C and 14,000 rpm for 15 minutes. Dilute 50 µL of the protein precipitation supernatant with 200 µL of aqueous 0.4 mM NH_4-Formate in LC vial.

6.1.2.5.3. Matrix-matched QC Standards (QC) at 3 Concentrations - Prepare additional standards from SD-1 (QC- High), SD-3 (QC – Medium), and SD-5 (QC – Low) as described in Section 6.1.2.5.2.

Note - Prepare enough QC Standards at the rate of 1 for every 10 samples or a minimum of 9 (whichever is greater) equally distributed between concentration levels.

6.1.2.6. Preparation of Samples

6.1.2.6.1. Spin sample (10, 6-dpf ZFL in ~ 500 µL water) in a centrifuge at low speed.

6.1.2.6.2. Remove as much of the 500 µL water as possible with a 200 µL pipette and 300 µL gel loading pipette tip.

6.1.2.6.3. Add ~ 100 mg (10 - 15 beads) 1.0 mm diameter (DIA) zirconia/silica beads to 2 mL tube.

6.1.2.6.4. Add 100 µL PFNA-1. Homogenize by 2, 20 second bursts at 6 m/s using FastPrep homogenizer.

6.1.2.6.5. Dilute with 400 µL of ISMIS-1 and vortex (protein precipitation).

6.1.2.6.6. Centrifuge at 4 °C and 14,000 rpm for 15 minutes.

6.1.2.6.7. Dilute 50 µL of the protein precipitation supernatant with 200 µL of aqueous 0.4 mM NH_4-Formate in LC vial.

Note – If the 100 µL homogenate concentration of an analyte is expected to be above the method range, 10 ng/mL – 1.25 µg/mL (20 ng/mL – 2.5

μg/mL GenX) in 100 μL homogenate, the homogenate can be diluted with additional PFNA-1; 100 μL of the diluted homogenate is then added to 400 μL of ISMIS-1 in a new tube for the rest of the sample preparation steps.

6.1.3. UPLC-MS/MS Method

The Thermo Vanquish UPLC and Orbitrap Fusion mass spectrometer instrument method uses the conditions identified in the following sections for the analysis of the 8 target analytes, the surrogate, and their respective ^{13}C-labeled internal standards.

6.1.3.1. UPLC Operating Conditions

Reservoirs A3: 0.4 mM ammonium formate in water with 5% ACN
Reservoirs B3: 0.4 mM ammonium formate in ACN with 5% H_2O
Column: BEH C18 reverse phase, 2.1x50 mm, 1.7 μm particle size
Flow Rate: 300 μL/min
Column Temperature: 50 °C
Injection Volume: 10 μL

Mobile Phase Gradient Program:

Time (min)	A3	B3	Curve
-3.00	80	20	Initial
0.00	80	20	Initial
0.05	80	20	5
2.00	50	50	5
3.00	50	50	5
3.10	40	60	5
4.00	40	60	5
5.00	15	85	5
5.10	0	100	5
7.00	0	100	5

6.1.3.2. Orbitrap Fusion Operating Conditions

The Thermo Orbitrap Fusion mass spectrometer will operate in negative-ion-spray (-ESI) with the following source general conditions:

Ion Source Parameters	
IonSource Type	H-ESI
Spray Voltage (V)	-3500
Sheath Gas (Arb)	25
Aux Gas (Arb)	6
Sweep Gas (Arb)	0
Ion Transfer Tube Temp. (°C)	300
Vaporizer Temp (°C)	30

The Thermo Orbitrap Fusion mass spectrometer will operate with the following conditions:

MS Parameters	
Application Mode	Standard
Default Charge State	1
Internal Mass Calibration	TRUE
Internal Mass Calibration Type	Easy-IC

The Thermo Orbitrap Fusion mass spectrometer will operate with 1 experiment using the following parameters:

Experiment 1 Prameters	
Start Time (min)	0
End Time (min)	6
Cycle Time (sec)	3

Master Scan - MS OT	
Detector Type	Orbitrap
Orbitrap Resolution	30000
Mass Range	Normal
Use Quadrapole Isolation	TRUE
Scan Range (m/z)	70 - 700
RF Lens (%)	60
AGC Target	4.00E+05
Max Injection Time (ms)	50
Microscans	1
Data Type	Profile
Polarity	Negative
Source Fragmentation	Disable

Targeted Mass List	
Compound	(m/z)
PFHxA	312.3973
PFHxS	398.9368
PFOA	412.9667
PFOS	498.9304
GenX	328.968
ADONA	376.9691
PFESA1	442.9267
PFNA	462.9635
GenX-CO2	284.9777
Apex Detection	
Expected Peak width (FWHM, s)	2
Desired Apex Window (%)	30
Dynamic Exclusion	
Exclude after n times	1
Exclusion duration (s)	60
Low Mass Tolerance (ppm)	10
High Mass Tolerance (ppm)	10
Exclude Isotopes	TRUE
Intensity	
Filter Type	ntensity Threshold
Intensity Threshold	2.50E+04
Data Dependent	
Data Dependent Mode	Cycle Time
Time Between Master Scans (s)	3
ddMS2 OT HCD Scan	
Isolation mode	Orbitrap
Isolation Window (m/z)	1.6
Isolation Offset	Off
Activation Type	HCD
HCD Collision Energy(%)	30
Stepped Collision Energy	TRUE
+/- HCD Collision Energy (%)	10
Detector Type	Orbitrap
Scan Range	Auto m/z Normal
Orbitrap Resolution	30000
First Mass (m/z)	75
ACG Target	5.00E+04
Inject Ions From Availible Parallelizable Time	TRUE
Max Injection Time (ms)	54
Microscans	1
Data Type	Centroid

6.1.4. Analysis by UPLC-MS/MS

6.1.4.1. Analysis of an Analytical Batch

The term analytical batch refers to all the standards, controls, and samples prepared together and analyzed together as one instrument sequence. Refer to Appendix 9.2 for an example of a sequence created in Xcalibur 4.1 for an analytical batch.

WARNING

Include a minimum of 3 injections of a ACN at the start of a sequence prior to the injection of the initial Solvent Blank to ensure the removal of system contaminants from preceding sample sets *and ensure equilibration of the UPLC-MS/MS and LC column to method conditions.*

6.1.4.1.1. Create the sample list in Xcalibur 4.1 to include the following standards and controls in addition to samples in the prescribed sequential order and at the described frequency:

- Minimum of 7 CS starting at the lower limit of quantitation (LLOQ) and covering the range of interest analyzed before any samples
- QC standards (low, medium, and high concentrations) are run at the rate of 1 for every 10 samples or a minimum of 9 (whichever is greater), QC samples are distributed equally at each concentration by multiples of 3
- BM control samples are run at a frequency of 1 per batch
- MB and LRB control samples are run at frequency of 1 for every 10 samples
- SB control samples are run prior to and immediately following CS, and immediately following all QC standards or groups of QC standards including the final QCs

6.1.4.1.2. Complete the sample list table:

- Standards and controls are named in the sample table by their abbreviations and concentrations (except in the case of blanks)
- Samples are named by the sample name provided in the chain of custody
- Create the file name using the year month day table increment file increment format; example 20180201-01-001 is the 1st data file of the 1st sample table run on 2/01/2018
- Save the sequence using the year month day table increment format; example 20180201-01 is the 1st sequence run on 2/01/2018

6.1.4.1.3. Run the analytical batch in the Xcalibur 4.1 sequence table by an instrument method that uses the parameters identified in Section 6.1.3.

6.1.5. Data Processing

6.1.5.1. Use the Xcalibur 4.1 sequence table to process the analytical batch saving the sequence with the extension "-RP01".

The Xcalibur processing method operates with the following general conditions:

General MS Data Processing Parameters	
Detector Type:	MS
Peak Detect:	ICIS
Filter:	FTMS - p ESI Full ms[70.0000-700.0000]
Trace:	Mass Range
Mass Tolerence (ppm)	10
Mass Precision (Decimals)	4
Calibration By	Internal Standard

The Xcalibur processing method generates MS1 extracted ion chromatograms (EIC) using the following m/z values for each target analyte and internal standard:

Components	Mass (m/z)	MS1 EIC Trace
13C2_PFHxA	314.9794	Quan
PFHxA_269	268.983	Qual
PFHxA_312	312.9731	Quan
13C3-GenX	286.9847 + 331.9776	Quan
GenX_285	284.9777	Qual
GenX_285+329	284.9777 + 328.9678	Quan
GenX_329	328.9678	Qual
13C4-PFOA	416.9799	Quan
PFOA_369	368.9764	Qual
PFOA_413	412.9667	Quan
ADONA_377	376.9691	Quan
ADONA_251	250.9759	Qual
13C2-PFHxS	401.9462	Quan
PFHxS_399	398.9369	Quan
PFHxS_400	399.9395	Qual
PFESA1_443	442.9267	Quan
PFESA1_444	443.9295	Qual
13C5-PFNA	467.98	Quan
PFNA	462.9635	Quan
13C4-PFOS	502.9434	Quan
PFOS_499	498.9305	Quan
PFOS_500	499.9328	Qual

6.1.5.2. Open the processed sequence in Quan browser and perform an initial data review (refer to Section 6.3, Evaluation Criteria, for acceptance criteria).

6.1.5.2.1. Inspect each extracted ion chromatogram and the integration of all peaks.

6.1.5.2.2. Review individual calibration for each target analyte.

6.1.5.2.2.1. Review the response as peak area.

6.1.5.2.2.2. Review calculated values and accuracy (%R) for individual target analytes in each calibration.

6.1.5.2.2.3. Review calibration correlation coefficient for each target analyte.

6.1.5.2.2.4. Take any corrective actions as specified for the CS in Section 6.4. Corrective Actions.

6.1.5.3. Save the Quan Browser results as a results file (.xqn) and export the results to an Excel 2016 (filename.xlsx) maintaining the filename with the extension "_Short".

6.1.5.4. Use the Excel file to calculate the following QC metrics (refer to Section 6.2 Quality Control and Section 6.6 Calculations) and create a QC Results Summary for the batch:

- Internal Standard (IS) Response
- Relative Retention Time (RRT) Ratio
- Qualifier/Quantifier (Qual/Quan) Ion Ratio
- Surrogate Accuracy
- QC Sample Accuracy and Precision

6.1.5.5. Use the Excel file to calculate observed concentrations of target analytes corrected for any dilutions, convert values to the desired units of measure specified by the project's QAPP, and create a Sample Results Summary for the batch.

6.1.6. Data Review

6.1.6.1. Use the Excel file to review the calculated QC metrics against the acceptance criteria listed in Section 6.3, Evaluation Criteria.

6.1.6.2. Complete any actions required in Section 6.4, Corrective Actions, in response to any QC metrics that exceed the acceptance criteria.

6.1.6.3. Complete any data review, verification, and validation protocols required by the QAPP of the project for which this SOP was used.

6.1.7. Results Report

Report final results for the batch as specified by the QAPP of the project for which this SOP was used.

6.2. Quality Control

6.2.1. Pipette Verification – Pipette performance is verified using 5 replicate measurements performed with the pipette set to its mid-range volume (pipette delivering volumes > 20 µL) or max volume (pipettes delivering volumes < 20 µL) to calculate Average % Difference and the %RSD for calculated volumes.

6.2.2. Matrix-matched Calibration Standards (CS) - The relationship between instrument response and concentration is demonstrated as continuous and reproducible for each analyte of interest using matrix-matched calibration standards. Minimum of 7, CS are prepared and run with every batch prior to the analysis of sample. CS cover the concentration range from the lower limit of quantitation (LLOQ) to the Upper Limit of Quantitation (ULOQ).

6.2.3. Quality Control (QC) Standards – QC standards are additional matrix-matched calibration standards prepared at 3 concentrations that will generate extracts with corresponding to low, medium, and high concentrations relative to the calibration standards. QC samples are used to assess method accuracy, precision, and the continued acceptance of the calibration for analytes. QC samples are run at the beginning and end of each batch and after every tenth sample. QC samples are distributed equally at each concentration by multiples of 3 with a minimum of 9 prepared with a batch.

6.2.4. Blanks – Blanks are used to demonstrate that the system is free from contamination and interference from the matrix and that the analytical method is able to differentiate and quantitate the analyte in the presence of other components of the sample.

6.2.4.1. Solvent Blanks (SB) – SB control sample is an aliquot of 2.5 mM ammonium acetate in 5% MeOH and water in an LC vial. SB are used to equilibrate the UPLC-MS prior to analysis of CS.

6.2.4.2. Laboratory Reagent Blanks (LRB) – LRB control samples are reagent water processed as a sample. LRB control samples are used to demonstrate that reagents and analysis system are free from contamination and that the UPLC-MS/MS does not experience carry-over contamination from injection to injection. LRB control samples are run at frequency of 1 for every 10 samples immediately following the last in each series of QC standards.

6.2.4.3. Method Blanks (MB) - MB control samples are 10% Hanks solution prepared and analyzed with internal standards as a sample. MB control samples are used to demonstrate that the entire preparation and analysis system is free from contamination. MB control samples are run at frequency of 1 for every 10 samples.

6.2.5. Perfluorononanoic Acid (PFNA) as a surrogate analyte is included in preparation of the LRB, MB, and all samples. The surrogate accuracy is used to demonstrate acceptable sample preparation and analysis performance.

6.2.6. Internal Standard (IS) Response - IS Response as peak area for each internal standard is monitored in all standards, controls, and samples. IS Response is used to monitor for gross failures during sample preparation and instrument performance.

6.2.7. Relative Retention Time (RRT) Ratio – The RRT ratio is a ratio of the retention time (RT) of the internal standard quantifier ion divided by the RT of the target analyte quantifier ion. RRT ratio is used to assign a qualitative identification of the target analyte. RRT ratio is monitored in all standards, controls, and samples.

6.2.8. Qualifier/Quantifier (Qual/Quan) Ion Ratio – the Qual/Quan ion ratio for target analytes is the peak area of the qualifier ion divided by the peak area of the quantifier ion. The Qual/Quan ion ratio is used to assign a qualitative identification of the target analyte. The Qual/Quan ion ratio is monitored in all standards, controls, and samples.

6.3. Evaluation Criteria

6.3.1. Pipettes

- 5 replicate measurements performed with the pipette set to its mid-range volume (pipette delivering volumes > 20 µL) or max volume (pipettes delivering volumes < 20 µL)

Pipette	Max Ave. % Diff	Max %RSD
Rainin Pipette-Litte XLS L10, S/N B619470071	10	10
Rainin Pipette-Litte XLS L20, S/N G1482996T	10	5
Rainin Pipette-Litte XLS L200, S/N G14827627	5	5
Rainin Pipette-Litte XLS L1000, S/N C15695857	5	5

6.3.2. Matrix-matched Calibration Standards (CS)

- Minimum of 7 calibration standards from LLOQ to the ULOQ
- The regression must have correlation coefficient (r^2) of 0.99 or greater
- Back calculated values for CS must be within +/- 20% of the nominal concentration (+/- 30% at the LLOQ)
- Response (peak areas) for target analytes and internal standards in calibration standards must not decrease by >50% of those observed during method validation.

Sample ID	PFHxA_312 Quan Ion Peak Area (counts)	PFHxS_399 Quan Ion Peak Area (counts)	PFOA_413 Quan Ion Peak Area (counts)	PFOS_499 Quan Ion Peak Area (counts)	GenX_285+329 Quan Ion Peak Area (counts)	ADONA_377 Quan Ion Peak Area (counts)	PFESA1_443 Quan Ion Peak Area (counts)	PFNA Quan Ion Peak Area (counts)
391 pg/mL Average Peak Area	5.2E+05	8.5E+05	2.9E+05	3.2E+05	2.4E+05	7.9E+05	8.8E+05	2.3E+05
391 pg/mL Peak Area Acceptance Limit	2.6E+05	4.3E+05	1.4E+05	1.6E+05	1.2E+05	3.9E+05	4.4E+05	1.2E+05
781 pg/mL Average Peak Area	8.8E+05	1.5E+06	4.3E+05	6.0E+05	4.0E+05	1.5E+06	1.6E+06	4.3E+05
781 pg/mL Peak Area Acceptance Limit	4.4E+05	7.5E+05	2.1E+05	3.0E+05	2.0E+05	7.4E+05	8.0E+05	2.1E+05
1.56 ng/mL Average Peak Area	1.7E+06	3.0E+06	8.6E+05	1.2E+06	7.7E+05	2.6E+06	3.1E+06	8.6E+05
1.56 ng/mL Peak Area Acceptance Limit	8.3E+05	1.5E+06	4.3E+05	6.0E+05	3.8E+05	1.3E+06	1.5E+06	4.3E+05
3.13 ng/mL Average Peak Area	3.2E+06	5.5E+06	1.5E+06	2.3E+06	1.5E+06	5.6E+06	5.6E+06	1.6E+06
3.13 ng/mL Peak Area Acceptance Limit	1.6E+06	2.7E+06	7.6E+05	1.1E+06	7.5E+05	2.8E+06	2.8E+06	8.0E+05
6.26 ng/mL Average Peak Area	6.2E+06	1.2E+07	3.6E+06	5.6E+06	3.0E+06	1.1E+07	1.2E+07	3.9E+06
6.26 ng/mL Peak Area Acceptance Limit	3.1E+06	6.1E+06	1.8E+06	2.8E+06	1.5E+06	5.3E+06	6.2E+06	2.0E+06
12.5 ng/mL Average Peak Area	1.1E+07	2.0E+07	6.0E+06	9.5E+06	5.6E+06	1.8E+07	2.0E+07	6.5E+06
12.5 ng/mL Peak Area Acceptance Limit	5.6E+06	9.9E+06	3.0E+06	4.7E+06	2.8E+06	9.2E+06	1.0E+07	3.2E+06
25 ng/mL Average Peak Area	2.1E+07	3.6E+07	1.1E+07	1.9E+07	1.0E+07	3.3E+07	3.6E+07	1.3E+07
25 ng/mL Peak Area Acceptance Limit	1.0E+07	1.8E+07	5.7E+06	9.6E+06	5.2E+06	1.7E+07	1.8E+07	6.7E+06
50 ng/mL Average Peak Area	3.5E+07	5.6E+07	1.9E+07	3.0E+07	1.8E+07	5.2E+07	5.5E+07	2.2E+07
50 ng/mL Peak Area Acceptance Limit	1.7E+07	2.8E+07	9.5E+06	1.5E+07	9.2E+06	2.6E+07	2.7E+07	1.1E+07
	PFHxA-13C2	PFHxS-13C3	PFOA-13C4	PFOS-13C4	GenX-13C3	PFOA-13C4	PFOA-13C4	PFNA-13C5
Validation Set Average Peak Area (counts)	9.75E+06	1.86E+07	6.21E+06	1.08E+07	3.09E+06	6.21E+06	6.21E+06	5.92E+06
Peak Area Acceptance Limit (counts)	4.88E+06	9.29E+06	3.10E+06	5.39E+06	1.54E+06	3.10E+06	3.10E+06	2.96E+06

6.3.3. Solvent Blanks (SB)

SB are used only to equilibrate the UPLC-MS/MS system and are not used to evaluate method or instrument performance.

6.3.4. Laboratory Reagent Blanks (LRB) and Method Blanks (MB)

- LRB and MB must report concentrations at or below the MDL for GenX, ADONA, PFOS, PFHxS, and Nafion, Compound 1
- LRB and MB must report concentrations at or below 1/3 the concentration of the LLOQ for PFOA and PFHxA (PFOA and PFHxA are known to be pervasive background contaminants)

Analyte	MDL - LC Vial (pg/mL)	1/3 LLOQ - LC Vial (pg/mL)	LLOQ - LC Vial (pg/mL)
PFHxA	39	130	391
PFHxS	27	NA	395
PFOA	32	130	391
PFOS	18	NA	397
GenX	62	NA	425
ADONA	25	NA	391
PFESA1 (Nafion, BP-1)	32	NA	390
PFNA (surrogate)	29	NA	395

6.3.5. Quality Control (QC) Standards (Low, Medium, and High)

- Back calculated values for a minimum of 1 QC standard bracketing a portion of a batch must be within 20% of the nominal concentration
- The average back calculated concentration must be within 20% of the theoretical concentration at each concentration for all target
- Precision less than 20% RSD at each concentration
- Minimum of 75% of the individual QC samples included with a batch must be within 20% of the theoretical concentration

6.3.6. Perfluorononanoic Acid (PFNA) Surrogate Analyte back calculated concentration must be within +/- 30% of the theoretical concentration

6.3.7. Internal Standard (IS) Response acceptance limit is set for each batch as 50% of the average IS peak area of the calibration standards run with the batch

6.3.8. Relative Retention Time (RRT) Ratio acceptance limits are set using the for the non-zero standards run with the batch. RRT Ratio acceptance limits are set as the average RRT +/- 3*RRT standard deviation.

6.3.9. Qualifier/Quantifier (Qual/Quan) Ion Ratio acceptance limits are set as (Qual/Quan) +/- (Qual/Quan)*0.2 for qualifier ions with > 50% response of quantifier ions for the non-zero standards run with the batch. Limits are set as (Qual/Quan) +/- (Qual/Quan)*0.5 for qualifier ions with < 50% response of quantifier ions for the non-zero standards run with the batch.

6.3.10. Data-dependent MS2 Spectra

- Data-dependent MS2 spectra for the Quan MS1 ion are manually reviewed for the presence of fragments (+/- 5 ppm)

  PFHxA (m/z) 268.9827
  PFHxS (m/z) 398.9358, 79.9571
  PFOA (m/z) 368.9759, 218.9859, 168.9891
  PFOS (m/z) 498.9296
  GenX (m/z) 184.9840, 168.9891, 118.9924
  ADONA (m/z) 250.9757, 84.9905
  PFESA1 (Nafion, BP-1) (m/z) 442.9258, 262.9758, 146.9874

6.4. Corrective Actions

6.4.1. Pipette Verification

- If a pipette fails to meet acceptance criteria repeat the verification process
- Pipettes that fail to meet specification after a second attempt are taken out of service and replaced by functioning pipettes until they can be serviced and meet the specifications for accuracy and precision

6.4.2. Matrix -matched Calibration Standards (CS)

- If response (peak areas) for target analytes and internal standards in calibration or CCV standards decrease by >50% of those observed during method validation, inspect and perform system maintenance on UPLC-MS/MS
- If one calibration standard at the lowest or highest concentration fails to meet accuracy requirements or the curve has an $r^2 < 0.99$ and more than 7

calibration standards were used the lowest or highest calibration standard may be excluded. The new LLOQ or ULOQ must be noted for sample results determined from the modified reporting range

- If neither of the 1st two actions result in calibration curve meeting the acceptance criteria, prepare and analyze a new set of calibration and CCV standards
- If none of the actions listed above results in calibration and CCV performance that meet method acceptance criteria, report the data with the appropriate data qualifiers listed below. Refer to Appendix 9.2, Data Qualifiers and Definitions, for a list of data qualifiers and definitions

    - If the calibration fails to meet acceptance criteria and the analyte is present qualify with J
    - If the calibration fails to meet acceptance criteria and the analyte is not present above the LLOQ qualify with UJ

6.4.3. Laboratory Reagent Blanks (LRB), and Method Blanks (MB)

- If target analytes are observed in the LRB, or MB at response/concentrations above method acceptance criteria, results are reported with the deviation noted in the sample report with the appropriate data qualifiers listed below. Refer to Appendix 9.2, Data Qualifiers and Definitions, for a list of data qualifiers and definitions

    - If the analyte is present in the LRB or MB exceeding acceptance criteria and the analyte is present qualify with J+
    - If the analyte is present in the LRB or MB exceeding acceptance criteria and the analyte is not present qualify with U

6.4.4. Quality Control (QC) Samples

- If all the QCs bracketing a portion of a batch fail to meet acceptance criteria, reanalyze the QCs
- If the reanalysis fails, prepare new calibration and QC standards and reanalyze all samples analyzed after the last acceptable QCs
- If < 75% of the QC meet the individual accuracy requirements and or the precision requirement is not met, evaluate the UPLC-MS/MS system, perform any required maintenance, and rerun the sample set (standards and samples)
- If accuracy and/or precision requirement are still not met, prepare new standards and controls and rerun the sample set
- If accuracy and/or precision requirement are still not met the results are reported with the accuracy and/or precision deviation noted in the sample report with the appropriate data qualifiers listed below. Refer to Appendix 9.2, Data Qualifiers and Definitions, for a list of data qualifiers and definitions

    - If the QC results fails to meet acceptance criteria by reporting both +/- 20% of the theoretical value and the analyte is present qualify with J
    - If the QC results fails to meet acceptance criteria by reporting +20% of the theoretical value and the analyte is present qualify with J+
    - If the QC results fails to meet acceptance criteria by reporting -20% of the theoretical value and the analyte is present qualify with J-
    - If the QC results fails to meet acceptance criteria by reporting -20% of the theoretical value and the analyte is not present qualify with UJ
    - If the QC results fails to meet acceptance criteria by >20% RSD and the analyte is present qualify with J
    - If the QC results fails to meet acceptance criteria by >20% RSD and the analyte is not present qualify with UJ

6.4.5. Perfluorononanoic Acid (PFNA) Surrogate Analyte

- If surrogate response does not meet the listed criteria, reinject the sample, controls, and bracketing QC standards
- If surrogate response still does not meet listed criteria, prepare and analyze a new LC sample (if possible) with controls, and bracketing QC standards
- If surrogate response still does not meet listed criteria results are reported with the surrogate recovery deviation noted in the sample report with the appropriate data qualifiers listed below. Refer to Appendix 9.2, Data Qualifiers and Definitions, for a list of data qualifiers and definitions

    - If the surrogate result fails to meet acceptance criteria by reporting >30% of the theoretical value and the analyte is present qualify with J+
    - If the surrogate result fails to meet acceptance criteria by reporting <30% of the theoretical value and the analyte is present qualify with J-

6.4.6. Internal Standard (IS) Response

- If IS response does not meet the listed criteria, reinject the sample, controls, and bracketing QC standards
- If IS response still does not meet listed criteria, prepare and analyze a new LC sample (if possible) with controls, and bracketing QC standards
- If IS response still does not meet listed criteria, the results are reported with the deviation noted in the sample report with the appropriate data qualifier listed below. Refer to Appendix 9.2, Data Qualifiers and Definitions, for a list of data qualifiers and definitions

    - If the IS response fails to meet acceptance criteria qualify with R

6.4.7. Relative Retention Time (RRT) Ratio

Results for a target analyte above the MDL which do not meet the criteria must be considered tentatively identified and noted accordingly in the sample report with the appropriate data qualifier listed below. Results for a target analyte below the MDL are not qualified based on RRT performance. The analyst can review and overrule cases for a standard, quality control sample, or sample where the analyte is expected to be present when the RRT qualitative identifier exceeds the acceptance criteria control limit but is within 1% of the average RRT for the set. Cases where the R data qualifier is overruled by the analyst are identified in the individual analyte Quality Control Report and research notebook with a narrative explanation, in these cases the R data qualifier is not transcribed to the final data summary table. Refer to Appendix 9.2, Data Qualifiers and Definitions, for a list of data qualifiers and definitions

- If the RRT fails to meet acceptance criteria and the reported concentration of the target analyte is above the MDL qualify with R
- Results for a target analyte below the MDL are not qualified based on RRT performance

6.4.8. Qualifier/Quantifier (Qual/Quan) Ion Ratio

Results for a target analyte reported above the MDL but below the LLOQ which do not meet the criteria must be considered tentatively identified and noted with the U data qualifier. Results for a target analyte reported above the LLOQ which do not meet the criteria must be considered tentatively identified and noted with the R data qualifier. Results for a target analyte reported below the MDL are not qualified based on Qual/Quan Ion Ratio performance. The analyst can review and overrule cases for a standard, quality control sample, or sample where the analyte is expected to be present when the Qual/Quan Ion Ratio qualitative identifier exceeds the acceptance criteria control limit. Cases where the data qualifier is overruled by the analyst are identified in the individual analyte Quality Control Report and research notebook with a narrative explanation, in these cases the data qualifier is not transcribed to the final data summary table. Refer to Appendix 9.2, Data Qualifiers and Definitions, for a list of data qualifiers and definitions

- If the Qualifier/Quantifier Ion Ratio fails to meet acceptance criteria and the analyte is present above the LLOQ qualify with R
- If the Qualifier/Quantifier Ion Ratio fails to meet acceptance criteria and the analyte is present above the MDL but below the LLOQ qualify with U
- Results for a target analyte reported below the MDL are not qualified based on Qual/Quan Ion Ratio performance

6.4.9. Data-dependent MS2 Spectra

Results for a target analyte which do not meet the acceptance criteria must be considered tentatively identified and noted accordingly in the sample report with the appropriate data qualifiers listed below. The analyst can review and overrule cases for a standard, quality control sample, or sample where the analyte is expected to be present when the data-dependent MS2 spectra qualitative identifier exceeds the acceptance criteria control limit. Cases where the data qualifier is overruled by the analyst are identified in the individual analyte Quality Control Report and research notebook with a narrative explanation, in these cases the data qualifier is not transcribed to the final data summary table. Refer to Appendix 9.2, Data Qualifiers and Definitions, for a list of data qualifiers and definitions

- If the data-dependent MS2 spectra fails to meet acceptance criteria and the analyte is present above the LLOQ qualify with R
- If the data-dependent MS2 spectra fails to meet acceptance criteria or is not collected by the instrument and the analyte is present below the LLOQ qualify with U
- Results for a target analyte reported below the MDL are not qualified based on MS2 Spectra

6.5. Recordkeeping

6.5.1. General Description of Records

Paper logbooks for the UPLC-MS/MS and balance are located adjacent the instruments. Entries relevant to sample analysis using this SOP are scanned to electronic records and entered in project's notebook.

Electronic records generated during sample analysis using this SOP for planning and documenting experiments will be stored in the project's file on the EPA\RTP network drive in accordance with the project's QAPP and ORD PPM 13.06. Electronic records include but are not limited to MS Excel spreadsheets, Word files, PDF files, text files, and OneNote files.

Xcalibur 4.1 software electronic records are created on Dell Optoplex XE2 Workstation (S/N H487KH2) located in D285-A in a project specific directory.

Sequence – the run table identifying sample names, raw data files, instrument methods, vial position, and injection volume. These are used to run the samples and process data. Sequences are located at

C:\Xcalibur\Data\Adam\(file named for the date of analysis)\sequncename.sld

Raw Data files – the raw and processed data generated by the UPLC-MS/MS during acquisition and processing. Raw data is located at

C:\Xcalibur\Data\Adam\(file named for the date of analysis)\filename.raw

Instrument Method Files – the files that contain the instrument operating parameters including those specified in this SOP. Instrument method files are located at

C:\Xcalibur\Methods\Adam\filename.meth

Processing Method Files – the files that contain data processing method parameters. They are used to generate extracted ion chromatograms, identify and integrate peaks, generate calibration curves, and calculate the observed concentration for target analytes. Processing Method files are located

C:\Xcalibur\Methods\Adam\filename.pmd

Quan Browser Result Files – the files that contain results (extracted ion chromatograms, peaks area, calibration curves, and observed concentration for target analytes) for processed data. Quan Browser result files are located

C:\Xcalibur\Data\Adam\(file named for the date of analysis)\

6.5.2. Custody and disposition of Records

The individual performing this SOP is responsible for documenting its use for analysis of a project's samples in a corresponding project notebook and the custody of that notebook for the duration of the project. The individual performing this SOP is responsible for generating and maintaining records created as a result of data processing, review, and reporting (Sections 6.1.4 – 6.1.6) for the duration of the project. At the completion of the project the individual performing this SOP is responsible for turning over all records to the project's lead/principal investigator.

6.6. Calculations

6.6.1. Accuracy as % Difference (%Diff)

$$\%Diff = 100\% * (Calculated\ Amt. - Theoretical\ Amt.)/Theoretical\ Amt.$$

6.6.2. Accuracy as % Recovery (%R)

$$\%R = 100\% * \frac{Reported\ Value}{Theoretical\ Value}$$

6.6.3. Precision as % Relative Standard Deviation (%RSD)

$$\%RSD = 100\% * \ standard\ deveiation/mean$$

6.6.4. Internal Standard (IS) Response

$$IS\ Response = 100\% * \frac{IS\ Response\ (Peak\ Area)}{CS\ Ave\ IS\ Response\ (Peak\ Area)}$$

6.6.5. Qualifier/Quantifier (Qual/Quan) Ion Ratio

$$\frac{Qual}{Quan}\ Ion\ Ratio\ (unitless) = \frac{Qual\ Ion\ Peak\ Area}{Quan\ Ion\ Peak\ Area}$$

6.6.6. Relative Retention Time (RRT) Ratio

$$RRT\ (unitless) = {IS\ Quan\ Ion\ RT\ (min)}\Big/{Analyte\ Quan\ Ion\ RT\ (min)}$$

6.7. Chain of Custody

Samples received for analysis by this SOP will be accompanied by a Chain of Custody (COC) document (Appendix 9.4, Example Chain of Custody Form) listing the samples with name, description, date of collection, and physical condition. The COC document will be provided in electronic format to both the releasing party and the receiving party. On the date of transfer, samples will be inspected against the COC list and any discrepancies noted on the COC. The COC will be signed and dated by the individuals receiving and relinquishing custody of the samples. Signed COCs will be scanned to generate an electronic copy in PDF format and added to a project's research notebook. The paper copies will be stored in the project file.

Samples are transferred under the conditions specified by a projects Quality Assurance Project Plan (QAPP). If samples are not immediately processed they are stored under the conditions specified by a projects QAPP. Samples that are not used in their entirety are returned to an individual specified by the project's Principle Investigator (PI) using the protocol described in the preceding paragraph or disposed of at the PI's request in accordance with the EPA/RTP Chemical Hygiene Plan[8.1].

7. Quality Control Rationale

Quality Control procedures, tests, and acceptance criteria specified for this SOP ensure the data generated are of known and verifiable quality.

7.1. Quality Control for Analytical Balance

Calibration and calibration checks ensure and document the accurate performance of the balance. The balance is operated and maintained according to OP-NHEERL-RCU-PRC-AES/2015-005-r00 OPERATING PROCEDURE FOR AE100 Analytical Balance

Operation, Calibration, and Maintenance[8.2]. The balance is calibrated prior to use and verified by pre and post-calibration verifications. Refer to the OP for a detailed description.

7.2. Quality Control for Pipettes

The proper function of all pipettes is necessary to ensure accurate and precise preparation of standards, controls, and samples. Rainin Pipette-Lite XLS adjustable pipettes are operated according the manufacturer's operating instructions[8.3]. The pipettes are serviced, evaluated for accuracy and precision, and certified annually by an external vendor. The proper function of all pipettes is evaluated and verified prior to their use for the preparation for any standard, control, or sample prepared as part of a method validation experiment or the analysis of actual samples.

The function of pipettes is evaluated for accuracy as % Difference and precision (%RSD) at 1 volume with 5 replicates using room temperature water and an analytical balance prior to their use. Pipettes that fail to meet specification are taken out of service and replaced by functioning pipettes until they can be serviced and meet for accuracy and precision.

7.3. Quality Control for Sample Preparation and Analysis with UPLC-MS/MS

Quality Control procedures, tests, and acceptance criteria specified for this SOP ensure the sample preparation and analysis provide data of known and verifiable quality.

Identification Confirmation, RRT, Qualifier/Quantifier Ion Ratio, and Data-dependent MS2 Spectra – Identity confirmation for each target analyte is provided by the RRT, Qualifier/Quantifier Ion Ratio, and MS2 spectra. The RRT and Qual/Quan ratios are calculated for each analyte in each standard, control, and sample. The data-dependent MS2 spectra are manually reviewed for the presence of diagnostic fragments of exact mass observed in standards.

Calibration and Calibration Standards- The UPLC-MS/MS is calibrated for each sample batch using a minimum of 7 calibration standards covering concentrations from the LLOQ to the ULOQ. The calibration is verified for continued acceptance by the analysis of a QC before and after all samples and at a minimum rate of 1 for every 10 samples.

Specificity/Selectivity/Bias and Blanks – Specificity, selectivity and contamination are evaluated with SB, BM, LRB, and MB control samples. BM control samples are run at a frequency of 1 per batch, MB and LRB control samples are run at frequency of 1 for every 10 samples. SB control samples are run prior to and immediately following CS, and immediately following all QC standards including the final QC.

Accuracy, Precision, and Quality Control Samples – Accuracy and precision are evaluated with QC samples. QC sample replicates at concentrations (low, medium, and high) are included at the rate of 1 for every 10 samples or a minimum of 9 (whichever is more).

Surrogate Recovery - Sample preparation and analysis performance are evaluated for each sample using a surrogate analyte

Sample Preparation, Instrument Performance, and Internal Standard (IS) Response – Gross failures during sample preparation and instrument performance are evaluated using the Internal Standard Response. Internal standards are included in every standard, control, and sample with the exception of the SB and BM.

Solution Stability – PFC concentrated stocks in organic solvents have been shown to be stable for at least 1 month when stored at ambient temperature. PFC concentrated stocks prepared in house are prepared from neat materials and labeled with a 1-month expiration date. PFC concentrated stocks acquired from a vendor are used and stored according the vendor's recommendations. Intermediate standards and dilute standards are prepared fresh from concentrated stocks for use in individual experiments during method development, validation, and for the analysis of individual batches.

8. Reference

8.1. EPA/RTP Chemical Hygiene Plan, [HYPERLINK "https://intranet.ord.epa.gov/sites/default/files/media/SHEM/RTP/2016_chemical_hygiene_plan_chp_final_website_version.pdf"]

8.2. NHEERL/RCU OP, OP-NHEERL-RCU-PRC-AES/2015-005-r00 OPERATING PROCEDURE FOR AE100 Analytical Balance Operation, Calibration, and Maintenance

8.3. Pipet-Lite xls +Operating Instruction Manual, Rainin/Mettler Toledo [HYPERLINK "https://www.mt.com/dam/RAININ/PDFs/UserManuals/manual-pipettes/pipet-lite-xls-plus-operating-instruction-manual.pdf"]

9. Appendices

9.1. Example of a Sample List table for a 20-sample batch

This is an example to demonstrate the order of standards, controls, and samples. The actual names of standards, controls, sample will differ from the example as will the actual file paths, data file names, and instrument method names.

File Name	Path	Instrument Method	Position	Inj Vol	Sample Name
20180817-01-001	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:A1	10	Acetonitrile
20180817-01-002	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:A2	10	Solvent Blank
20180817-01-003	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:A3	10	CS, 391 pg/mL PFAS in 6-dpf ZFL
20180817-01-004	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:A4	10	CS, 781 pg/mL PFAS in 6-dpf ZFL
20180817-01-005	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:A5	10	CS 1.56 ng/mL PFAS in 6-dpf ZFL
20180817-01-006	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:A6	10	CS, 3.13 ng/mL PFAS in 6-dpf ZFL
20180817-01-007	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:A7	10	CS, 6.26 ng/mL PFAS in 6-dpf ZFL
20180817-01-008	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:A8	10	CS, 12.5 ng/mL PFAS in 6-dpf ZFL
20180817-01-009	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:A9	10	CS, 25 ng/mL PFAS in 6-dpf ZFL
20180817-01-010	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:B1	10	CS, 50 ng/mL PFAS in 6-dpf ZFL
20180817-01-011	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:B2	10	Lab Reagent Blank
20180817-01-012	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:B3	10	CS & QC - L, 1.56 ng/mL PFAS in 6-dpf ZFL
20180817-01-013	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:B4	10	CS & QC-M, 6.26 ng/mL PFAS in 6-dpf ZFL
20180817-01-014	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:B5	10	CS & QC-H, 25 ng/mL PFAS in 6-dpf ZFL
20180817-01-015	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:B6	10	Lab Reagent Blank
20180817-01-016	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:B7	10	MB, 6-dpf ZFL
20180817-01-017	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:B8	10	Sample-001
20180817-01-018	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:B9	10	Sample-002
20180817-01-019	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:C1	10	Sample-003
20180817-01-020	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:C2	10	Sample-004
20180817-01-021	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:C3	10	Sample-005
20180817-01-022	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:C4	10	Sample-006
20180817-01-023	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:C5	10	Sample-007
20180817-01-024	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:C6	10	Sample-008
20180817-01-025	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:C7	10	Sample-009
20180817-01-026	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:C8	10	Sample-010
20180817-01-027	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:C9	10	CS & QC - L, 1.56 ng/mL PFAS in 6-dpf ZFL
20180817-01-028	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:D1	10	CS & QC-M, 6.26 ng/mL PFAS in 6-dpf ZFL
20180817-01-029	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:D2	10	CS & QC-H, 25 ng/mL PFAS in 6-dpf ZFL
20180817-01-030	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:D3	10	Lab Reagent Blank
20180817-01-031	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:D4	10	MB, 6-dpf ZFL
20180817-01-032	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:D5	10	Sample-011
20180817-01-033	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:D6	10	Sample-012
20180817-01-034	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:D7	10	Sample-013
20180817-01-035	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:D8	10	Sample-014
20180817-01-036	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:D9	10	Sample-015
20180817-01-037	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:E1	10	Sample-016
20180817-01-038	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:E2	10	Sample-017
20180817-01-039	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:E3	10	Sample-018
20180817-01-040	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:E4	10	Sample-019
20180817-01-041	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:E5	10	Sample-020
20180817-01-042	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:E6	10	CS & QC - L, 1.56 ng/mL PFAS in 6-dpf ZFL
20180817-01-043	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:E7	10	CS & QC-M, 6.26 ng/mL PFAS in 6-dpf ZFL
20180817-01-044	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:E8	10	CS & QC-H, 25 ng/mL PFAS in 6-dpf ZFL
20180817-01-045	C:\Xcalibur\Data\Adam\20180817	C:\Xcalibur\methods\Adam\20180814-01_MS1+DDA_HCD	R:E9	10	Lab Reagent Blank

9.2. Data Qualifiers and Definitions

Data Qualifier	Definition
U	The analyte was analyzed for, but was not detected above the LLOQ.
J	The analyte was positively identified. The result is an estimated quantity. The associated numerical value is the approximate concentration of the analyte in the sample
J+	The analyte was positively identified.The result is an estimated quantity, but the result may be biased high.
J-	The analyte was positively identified.The result is an estimated quantity, but the result may be biased low.
UJ	The analyte was analyzed for, but was not detected. One or more acceptance criteria were exceeded. The reported LLOQ is approximate and may be inaccurate or imprecise. Further information will be included in the data report providing details of the failure.
R	The data are unusable. The sample results are rejected due to serious deficiencies in meeting QC criteria. The analyte may or may not be present in the sample.
M	Manual integration performed.

9.3. Chain of Custody

Chain of Custody Form

File: 20170327_Tal Lab_Chain_of_Custody.xls

(Chain of custody)	(Print Name)	(Date)	(Signature)
Relinquished by:	Tara Catron	3/27/2017	
Received by:	Adam Swank	3/27/2017	
Project Information			
Principal Investigator:	Tamara Tal		
QAPP #:	IRP-NHEERL-RTP/ITD/GCTB/TT/2015-01-0; & QAPP E-ADH-0030561-QP-1-0		
Total Number of Unique Samples:	12		
Total Number of Sample Containers:	12		
Physical Condition of Samples (example: frozen liquid at -80° C):	frozen at -80°C		
Sample Description:	zebrafish larvae (~10/tube)		

Sample Information							
Sample Name	Total Number of Containers per Sample	Total Sample Volume or Mass (units)	Sample Matrix	Sample Description	Collection Time (Date/Time/Etc.)	Container Type	Notes
MB70 Tal Lab-1/A-C	1	~10 larvae per container	zebrafish larvae, cell culture water (~ 200 uL)	CR larvae Dosed on Days 1, 6, 7, 8, 9 with 0.1% DMSO	3/10/2017	microcentrifuge tube	
MB70 Tal Lab-2/A-C	1	~10 larvae per container	zebrafish larvae, cell culture water (~ 200 uL)	CR larvae 1 with 0.1% DMSO	3/10/2017	microcentrifuge tube	
MB70 Tal Lab-3/A-C	1	~10 larvae per container	zebrafish larvae, cell culture water (~ 200 uL)	CR larvae 9 with 0.1% DMSO	3/10/2017	microcentrifuge tube	
MB70 Tal Lab-4/A-C	1	~10 larvae per container	zebrafish larvae, cell culture water (~ 200 uL)	CR larvae 6, 7, 8, 9 with 0.1% DMSO	3/10/2017	microcentrifuge tube	
MB70 Tal Lab-5/A-C	1	~10 larvae per container	zebrafish larvae, cell culture water (~ 200 uL)	CR larvae 6, 7 with 0.1% DMSO	3/10/2017	microcentrifuge tube	
MB70 Tal Lab-6/A-C	1	~10 larvae per container	zebrafish larvae, cell culture water (~ 200 uL)	CR larvae 8, 9 with 0.1% DMSO	3/10/2017	microcentrifuge tube	
MB70 Tal Lab-7/A-C	1	~10 larvae per container	zebrafish larvae, cell culture water (~ 200 uL)	CR larvae 1, 6, 7, 8, 9 with 0.3 uM triclosan	3/10/2017	microcentrifuge tube	
MB70 Tal Lab-8/A-C	1	~10 larvae per container	zebrafish larvae, cell culture water (~ 200 uL)	CR larvae 1 with 0.3 uM triclosan	3/10/2017	microcentrifuge tube	
MB70 Tal Lab-9/A-C	1	~10 larvae per container	zebrafish larvae, cell culture water (~ 200 uL)	CR larvae 9 with 0.3 uM triclosan	3/10/2017	microcentrifuge tube	
MB70 Tal Lab-10/A-C	1	~10 larvae per container	zebrafish larvae, cell culture water (~ 200 uL)	CR larvae 6, 7, 8, 9 with 0.3 uM triclosan	3/10/2017	microcentrifuge tube	
MB70 Tal Lab-11/A-C	1	~10 larvae per container	zebrafish larvae, cell culture water (~ 200 uL)	CR larvae 6, 7 with 0.3 uM triclosan	3/10/2017	microcentrifuge tube	
MB70 Tal Lab-12/A-C	1	~10 larvae per container	zebrafish larvae, cell culture water (~ 200 uL)	CR larvae 8, 9 with 0.3 uM triclosan	3/10/2017	microcentrifuge tube	

Sample	Flask/Replicate	Status	Dose	Day of Dosing	Experiment		
1	1/A-C	CR	0.1% DMSO	1, 6, 7, 8, 9	MB70		
2	2/A-C	CR	0.1% DMSO	1	MB70		
3	3/A-C	CR	0.1% DMSO	9	MB70		
4	4/A-C	CR	0.1% DMSO	6, 7, 8, 9	MB70		
5	5/A-C	CR	0.1% DMSO	6, 7	MB70		
6	6/A-C	CR	0.1% DMSO	8, 9	MB70		
7	7/A-C	CR	0.3 uM triclosan	1, 6, 7, 8, 9	MB70		
8	8/A-C	CR	0.3 uM triclosan	1	MB70		
9	9/A-C	CR	0.3 uM triclosan	9	MB70		
10	10/A-C	CR	0.3 uM triclosan	6, 7, 8, 9	MB70		
11	11/A-C	CR	0.3 uM triclosan	6, 7	MB70		
12	12/A-C	CR	0.3 uM triclosan	8, 9	MB70		